The Effects of Dietary Administration of Vitamin D on UVB Induced Skin Inflammation and Carcinogenesis

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for graduation *with distinction* in Biomedical Science in the undergraduate colleges of The Ohio State University

by

Amanda R. Harper

The Ohio State University
April 2010

Project Advisor: Professor Tatiana M. Oberyszyn, Department of Pathology

Abstract

In today's world, many people are deficient in vitamin D. Increased sun exposure acts as a mechanism to increase vitamin D levels, and studies have shown that optimal amounts of vitamin D can reduce the risk of internal cancers. However, the ultraviolet activation spectra for vitamin D photosynthesis, DNA damage and skin cancer are identical. Previous evidence has not revealed whether or not increased vitamin D levels play a protective role against the development of non-melanoma skin cancer. For this reason, we **hypothesized** that dietary vitamin D may inhibit UVB induced inflammation, a precursor to skin cancer, and tumor formation. In order to test this hypothesis, 150 male and female Skh-1 hairless mice were placed into groups receiving 1 of 3 diets containing 25 IU/g, 150 IU/g or 1000 IU/g vitamin D. In the acute study, mice were fed vitamin D diets for 4 weeks and were then exposed to one minimal erythemic dose (2240 J/m^2) of UVB light and sacrificed 48 hours following exposure while for the chronic study they were exposed three times weekly for 25 weeks. Inflammatory parameters including edema formation, myeloperoxidase activity, and skin morphology were examined in the acute study. Immunohistochemical analysis was also performed to detect the presence of cyclobutane pyrimidine dimers and p53. In the chronic study, tumors were measured over time and were graded by a veterinary pathologist at the cessation of the study. Vitamin D appeared to have no effect on the acute inflammatory response and dose dependently decreased direct DNA damage; however, p53 analysis revealed a possible dose dependent increase in global DNA damage. In the chronic study, male mice exhibited a vitamin D dose dependent increase in tumor size, burden, and grade. Together, these results suggest that dietary vitamin D supplementation may not be a viable option for prevention of cutaneous cancer.

Introduction

Non-melanoma skin cancer (NMSC) has the highest incidence of all cancer types, and the number of cases approximately equals that of all other human cancers combined [1]. For example, in 2010 alone, it is estimated that there will be over one million new cases of NMSC in the United States [2]. Moreover, the cost burden for treating this disease is substantial, and $426,000,000 is paid each year in order to efficiently and effectively treat NMSC in Medicare patients alone [3]. NMSC affects the lives of nearly all Americans in some way or another, and a great deal of research is aimed towards understanding the disease and its risk factors, as well as discovering potential therapeutics for patients suffering from NMSC.

There are two types of NMSC: basal cell and squamous cell carcinoma. While basal cell carcinoma accounts for eight out of every ten non-melanoma skin cancers, it is the least aggressive form of the disease. Alternatively, 20% of skin cancers are classified as the more aggressive NMSC, squamous cell carcinoma [1]. Figure 1 depicts key components of the skin, including the cell types found in the epidermal, dermal, and subcutis layers of the skin. Keratinocytes, including both squamous and basal cells, are the primary cell type residing in the outermost layer of the skin, the epidermis. These cells proliferate in the lower layers of the epidermis and differentiate as they migrate outward where they eventually die and are shed. However, the location of keratinocytes makes them particularly susceptible to the damaging effects of ultraviolet radiation. This can lead to harmful mutations in tumor suppressor genes which subsequently inhibit terminal differentiation of these cells. As a result, genetically altered cells do not follow the typical trajectory of a keratinocyte, and they are able to continue living and potentially become cancerous [4].

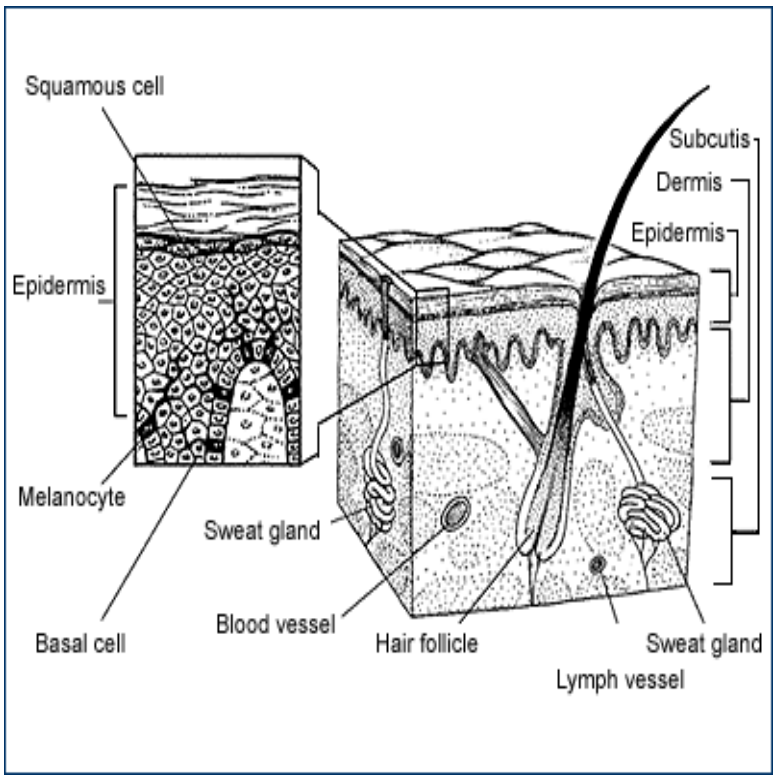

Figure 1. Diagram of the skin
Image courtesy of the American Cancer Society: http://www.cancer.org, (2006).

Ultraviolet radiation has been classified as the single most important risk factor for the development of non-melanoma skin cancer [5]. It is comprised of three different wavelengths including UVA, UVB, and UVC light. Ultraviolet light C induces the most genetic damage to cells upon exposure; however this form of radiation is primarily filtered out by the earth's atmosphere. In comparing the two wavelengths which may be detrimental to humans, ultraviolet light A and ultraviolet light B, UVA is nearly twenty times more abundant at the earth's surface; however, this form of radiation is not readily absorbed by DNA [6]. Thus, ultraviolet light B, with a wavelength ranging from 290 nanometers to 320 nanometers, has been labeled as the major environmental carcinogen that can lead to the development of both basal cell carcinomas and squamous cell carcinomas.

UVB radiation affects keratinocytes by initiating cellular DNA damage through primary and secondary mechanisms. Primary damage results after DNA has absorbed UVB light and the formation of cyclobutane pyrimidine dimers has occurred [7]. Following the initial exposure, the inflammatory response is exacerbated, thereby stimulating oxidative stress which results in secondary DNA damage. This series of UVB-induced cellular inflammatory responses has been shown to have a causal effect in the development of tumors in NMSC [8].

Inflammation, the pre-cursor to tumorigenesis in the skin, occurs following exposure to ultraviolet radiation. As part of the immune response to this carcinogen, neutrophils and other innate immune cells are activated and are directed towards the location of cellular damage through a mediating current of pro-inflammatory chemokines and cytokines within the epidermis. Once innate immune cells arrive at a particular point, they are thought to be

the primary sources of reactive oxygen species which enhance the inflammatory response and lead to secondary DNA damage in keratinocytes [9]. As a result of chronic UVB exposure, over time, DNA repair mechanisms are unable to effectively mend all of the damaged sites. Thus, DNA damage leads to mutations in DNA and the development of pre-cancerous cells which may eventually progress to cancerous lesions in the skin [10].

It is known that UVB-induced inflammation is a major risk factor for the development of non-melanoma skin cancer, and the most appropriate methods for preventing this disease include the usage of sunscreen and reducing sun exposure. However, countless people do not take heed of these recommendations, and alternative prevention and treatment mechanisms are necessary [11]. In the quest towards developing new approaches to non-melanoma skin cancer, the role of vitamin D has been called into question. Vitamin D is an essential nutrient, and though it can be found in numerous dietary sources, the primary source of vitamin D in humans is generated during exposure to sunlight. Hence, we have asked, is it possible, because ultraviolet light is a major player in the development and progression of NMSC, that vitamin D may also contribute substantially?

When ultraviolet light comes into contact with the skin, 90% of all vitamin D in the body is produced, making sunlight a crucial factor in preventing vitamin D deficiency [12]. Vitamin D deficiency in children may lead to rickets and growth failure resulting from a cascade of low vitamin D levels, increased release of parathyroid hormone, enhanced calcium mobilization from the bone, and eventual bone resorption. Adults who are deficient in vitamin D may also have issues concerning phosphate and magnesium metabolism as well as calcium absorption in the intestinal wall. Though vitamin D-fortified milk and other foods rich in vitamin D including cod liver oil may be consumed in order to promote a healthy

vitamin D status, it has been suggested that some other form of supplementation must be utilized, whether it be through intake of a daily multivitamin or increased ultraviolet light exposure [13].

In order to generate the most abundant source of vitamin D in humans, endogenous vitamin D synthesis occurs when UVB photons penetrate the skin and convert 7-dehydrocholesterol to pre-vitamin D_3 which is rapidly shuttled to the blood stream where it is later isomerized in the kidney and liver to vitamin D_3 [14]. This biologically active metabolite, more formally known as 1, 25-dihydroxyvitamin D_3 [1, 25$(OH)_2D_3$], assists in the execution of a variety of physiological processes throughout the body [15]. Additionally, studies have shown that vitamin D can expand its benefits by potentially inhibiting the development and/or progression of internal cancers. After binding to vitamin D receptors on cancer cells, it has been found to play a role in the regulation of cellular proliferation and differentiation in addition to the inhibition of angiogenesis [16]. However, the systemic levels of vitamin D required to achieve this potentially anti-carcinogenic effect are currently unknown.

Previous research has revealed that optimal amounts of vitamin D not only lead to reduced incidence of internal cancers but also play a role in the prevention of conditions such as bone, autoimmune, and cardiovascular disease. As a result, it has been suggested that increasing exposure to the primary source of vitamin D, sunlight, may be beneficial [17]. The ultraviolet activation spectra for vitamin D photosynthesis and that of DNA damage and skin cancer are identical [18]. However, because vitamin D is quickly transported from the skin to the bloodstream, we **hypothesized** that exogenous dietary vitamin D may be effective at inhibiting ultraviolet radiation induced inflammation and skin carcinogenesis. To test this

hypothesis, Skh-1 hairless mice were maintained on chow diets containing increasing doses of vitamin D while being exposed to UVB in both an acute and chronic study. At the cessation of the studies, mice were sacrificed and skin, tumors (in the chronic study only), and serum samples were acquired. Following extensive experimentation, we have determined that increasing dietary vitamin D supplementation may not be effective at inhibiting UVB induced inflammation or tumorigenesis in the skin.

Materials and Methods

Animal Treatments: Skh-1 hairless mice (Charles River Laboratories, Wilmington MA) were used for both the acute and chronic studies and were housed in the vivarium at The Ohio State University according to the requirements established by the American Association for Accreditation of Laboratory Animal Care. Prior to beginning all studies, procedures were approved by the appropriate Institutional Animal Care Utilization Committee.

Mice were housed five per cage. Animals were four weeks of age upon arrival at which point they were put on diets containing either 25IU/g (sub-physiologic), 150IU/g (physiologic), or 1000IU/g (supra-physiologic) Vitamin D3 (Research Diets). For each study, 25 males and 25 females were fed each diet, for a total of 150 mice per study. Diets were replaced three times weekly and average food intake per cage was determined. For the acute study, fifteen males and fifteen females per diet were exposed dorsally to one minimal erythemic dose of UVB (2240 J/m^2 as determined by a UVX radiometer (UVP Inc., Upland, CA)) emitted by Phillips FS40UVB lamps (American Ultraviolet Company, Lebanon, IN) that were fitted with Kodacel filters (Eastman Kodak, Rochester, NY) to ensure the emission of primarily UVB light (290-320nm). Mice were sacrificed 48 hours following irradiation. Ten males and ten females per diet served as age matched, unirradiated control mice. For the chronic study, fifteen males and fifteen females per diet were irradiated with three times weekly doses of 2240 J/m^2 UVB. Mice were irradiated for 25 weeks and sacrificed 24 hours following the final UVB exposure. As in the acute study, ten males and ten females per diet served as age matched, unirradiated control mice for the chronic study.

Please see Figure 2 for a more detailed acute and chronic study schematic.

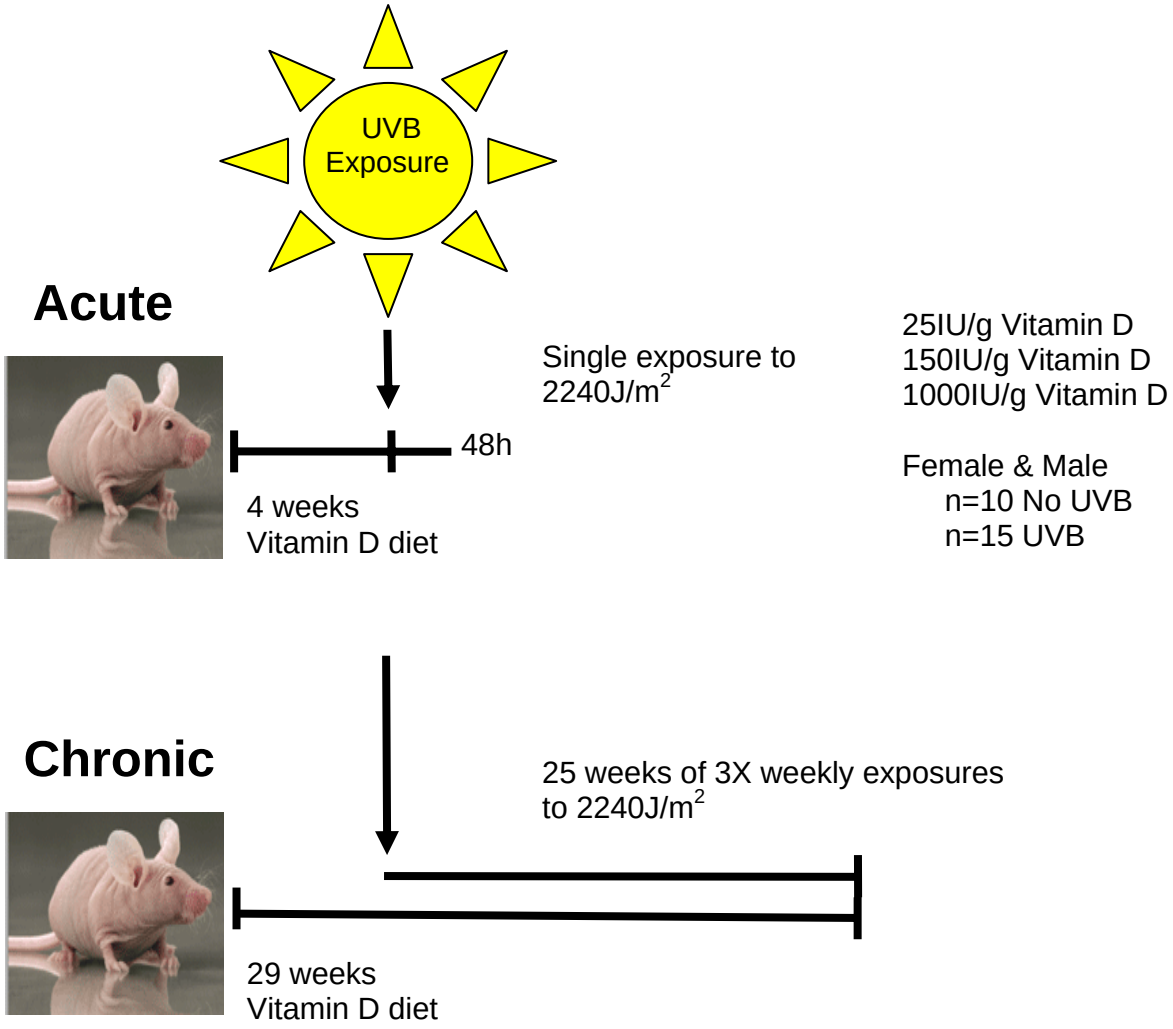

Figure 2. Vitamin D acute and chronic study schematic.

Quantitation of edema formation: Edematous response to acute UVB exposure was determined by measuring changes in dorsal skin fold thickness using digital calipers. Measurements were taken 48 hours after UVB exposure, which has been determined to be the peak of the UVB-induced inflammatory response.

Quantitation of tissue myeloperoxidase levels: Myeloperoxidase activity was determined biochemically in acutely irradiated skin. Myeloperoxidase (MPO) is an enzyme released by activated neutrophils in the inflammatory response which converts hydrogen peroxide to hypochlorous acid. The levels of MPO in cutaneous tissue were determined biochemically in order to quantify the amount of activated neutrophil infiltration. Skin punches were isolated from all mice with a 10-mm-diameter cork borer. Each punch was homogenized separately in 1.25 ml of 0.5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer, pH 6.0. The skin was homogenized at 4°C and subjected to three cycles of sonification, freezing, and thawing. The samples were then centrifuged for 15 minutes at 13,000 rpm at 4°C. The supernatants were transferred to individual wells of a 96-well microtiter plate with 290µl of substrate (0.167 mg/ml *o*-dianisidine dihydrochloride and 0.0005% H_2O_2 in 50 mM potassium phosphate buffer, pH 6.0). MPO activity was measured spectrophotometrically over a five minute period at 450 nm with a programmable microplate reader (Molecular Devices, Menlo Park, CA). The data are expressed as mean units of MPO activity, where the amount of MPO required to degrade 1 µmol of peroxidase/min at 25°C is equal to 1 U of MPO activity. MPO activity in each sample was calculated based on a standard curve.

Quantitation of epidermal and dermal skin thickness: Epidermal and dermal tissue morphology of male and female mice was analyzed histochemically. Immediately following sacrifice, 0.5 cm^2 skin section were placed in 10% neutral buffered formalin for two hours, washed with phosphate buffered saline solution (PBS), processed, and embedded in paraffin blocks. Tissue sections (5 μm) were cut and mounted onto Superfrost Plus microscope slides (Fischer Scientific). The tissue sections were then deparaffinized using Clear-Rite 3 (Richard-Allan Scientific, Kalamazoo, MI) and rehydrated in graded series of alcohols with a final wash in distilled water. Epidermal and dermal thicknesses were measured using ImageJ software at a magnification of 10x in all tissue samples.

Masson's Trichrome Staining: Reagents for the Accustain trichrome stain (Masson) kit (Sigma Chemical Co., St. Louis, MO) were utilized for the Masson's trichrome staining procedure. Rehydrated tissue sections were incubated for fifteen minutes in preheated Bouin's solution (56°C). The slides were then cooled in distilled water for two minutes, washed in distilled water, and stained for five minutes in Weigert's iron hematoxylin solution. After washing again in distilled water, the sections were stained with Beibrich scarlet-acid fuchsin for five minutes, washed in distilled water, and stained for five minutes in phosphotungstic/phosphomolybdic acid solution and then aniline blue solution. After incubating for two minutes in 1% acetic acid, the tissues were dehydrated and mounted.

Quantitation of cyclobutane pyrimidine dimers (CPD): Direct DNA damage, as measured by the presence of CPD in the epidermis, was quantified immunohistochemically. Rehydrated tissue sections were incubated in 0.3% H_2O_2 in 100% methanol for ten minutes at

room temperature, at 37°C in 0.125% trypsin for ten minutes, and in 1 N HCl for 30 minutes at room temperature, washed with distilled water rinses before and after each step. Sections were then blocked with M.O.M. (Vector Laboratories, Burlingame, CA) reagents according to kit instructions, followed by incubation with the primary anti-thymine dimer antibody (1:50, Kamiya Biomedical Co., Seattle, WA). Sections were then incubated with biotinylated secondary antibody followed by ABC reagent (Vector Laboratories) for 30 minutes each at room temperature. Finally, sections were incubated with diaminobenzadine (DAB) solution (Vector Laboratories) for 30 seconds, rinsed with distilled water, counterstained with hematoxylin 2 (Richard-Allan), dehydrated, and mounted. CPD-stained sections were analyzed by counting the number of positive and total nuclei in the epidermis from each mouse in 5 fields at a magnification of 60x. The data are expressed as the percentage of positive cells per field.

Quantitation of p53: Rehydrated tissue sections were incubated in 10% goat serum in PBS in order to block any nonspecific binding. The tissues were incubated in rabbit anti-p53 antibody (Vector Laboratories, Burlingame, CA) at room temperature for one hour. Vector Link and Label were then used to tag the primary antibody with HRP, and diaminobenzidine was used to visualize the antigen. p53-stained sections were analyzed by counting the number of positive and total nuclei in the epidermis from each mouse in 5 fields at a magnification of 60x. The data are expressed as the percentage of positive cells per field.

Tumor development: Tumor multiplicity, burden, and average size were determined weekly. Beginning at six weeks following the initial UVB exposure, neoplastic lesions located on the

dorsal skin measuring greater than 1 mm in size were counted and measured (length x width). Tumors were measured using digital calipers throughout the duration of the study.

Hematoxylin and Eosin Staining: Immediately following sacrifice, small, medium, and large representative tumors were placed in 10% neutral buffered formalin for two hours, washed with phosphate buffered saline solution (PBS), processed, and embedded in paraffin blocks. Tissue sections (5 μm) were cut and mounted onto Superfrost Plus microscope slides (Fischer Scientific). The tissue sections were then deparaffinized using Clear-Rite 3 (Richard-Allan Scientific, Kalamazoo, MI) and rehydrated in graded series of alcohols with a final wash in distilled water. After rehydration, skin tumor sections were stained for one minute in hematoxylin 2 (Richard-Allan Scientific). Following a two-minute wash in distilled water, the slides were dipped into Clarifier 2 (Richard-Allan Scientific), water rinsed for 30 seconds, dipped ten times in ammonia water, and then rinsed again for 30 seconds in water. After ten dips in 70% alcohol, the tissue was stained for 30 seconds in eosin-Y (Richard-Allan Scientific), dehydrated in a series of alcohols, and placed in Clear-Rite 3 (Richard-Allan Scientific) for ten minutes before mounting.

Tumor grade: Tumor grade was determined by a board certified veterinary pathologist. After sacrifice, three random tumors per irradiated mouse were harvested for histologic grading. Hematoxylin and Eosin-stained tissue sections of tumors isolated from UVB-irradiated male and female mice were graded in a blinded manner by board-certified veterinary pathologist Dr. Donna Kusewitt. Grades were assigned as follows: pre-cancerous, including hyperplasia and papilloma (grades 1–3), or cancerous, including microinvasive

squamous cell carcinoma (grades 1–3) or fully invasive squamous cell carcinoma. Papillomas were exophytic tumors that showed no evidence of stromal invasion, whereas squamous cell carcinomas had a more endophytic appearance, with stromal invasion evidenced by loss of basement membrane continuity and development of an inflammatory stromal response. A grade 1 papilloma was composed primarily of epithelium without a pronounced papillary pattern; a grade 2 papilloma was a well-differentiated papillary mass; and a grade 3 papilloma was similar to a grade 2 papilloma, except that a few finger-like projections of atypical cells at the base of the mass were present. Microinvasive squamous cell carcinomas were subcategorized by depth of penetration into the dermis. Only tumors that invaded the panniculus carnosus were classified as fully invasive squamous cell carcinomas. Hyperplasia and all grades of papilloma were considered benign, whereas squamous cell carcinoma and all grades of microinvasive squamous cell carcinoma were considered malignant.

Statistical Analysis: Statistical analysis was performed to analyze the significance of both acute and chronic study data. Two-sample *t* tests were performed using Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA) to compare skin fold thickness, myeloperoxidase activity, epidermal thickness, dermal thickness, CPD, p53, tumor size and tumor burden between genders. For all comparisons, a two-sided $p \leq 0.05$ level of significance was used.

Results

Effects of vitamin D on acute UVB induced cutaneous edema

In analyzing the effects of vitamin D on acute UVB induced edema (Fig. 3), digital calipers were used to measure dorsal skin fold thickness (in mm). As part of the inflammatory response, edema formation can be quantified in order to provide insight to the intensity of the response to UVB radiation. In this experiment, in the absence of UVB radiation, male mice exhibited a significantly larger skin fold thickness in comparison to females regardless of dietary vitamin D levels. Following exposure to ultraviolet light, skin fold thickness in the experimental groups increased significantly relative to the unirradiated control mice. These measurements reveal that vitamin D did not play a role in UVB mediated edema formation.

Effects of vitamin D on acute UVB induced cutaneous myeloperoxidase (MPO) activity

MPO activity was also quantified to determine the effects of vitamin D on neutrophil activation as part of the inflammatory response (Fig. 4). Following exposure to UVB light, both male and female mice expressed increased myeloperoxidase levels compared to unirradiated control mice. In both the 25 IU/g and 1000IU/g diets, female MPO levels were significantly higher than male MPO levels. However, within each gender, vitamin D had no effect on UVB induced myeloperoxidase activity.

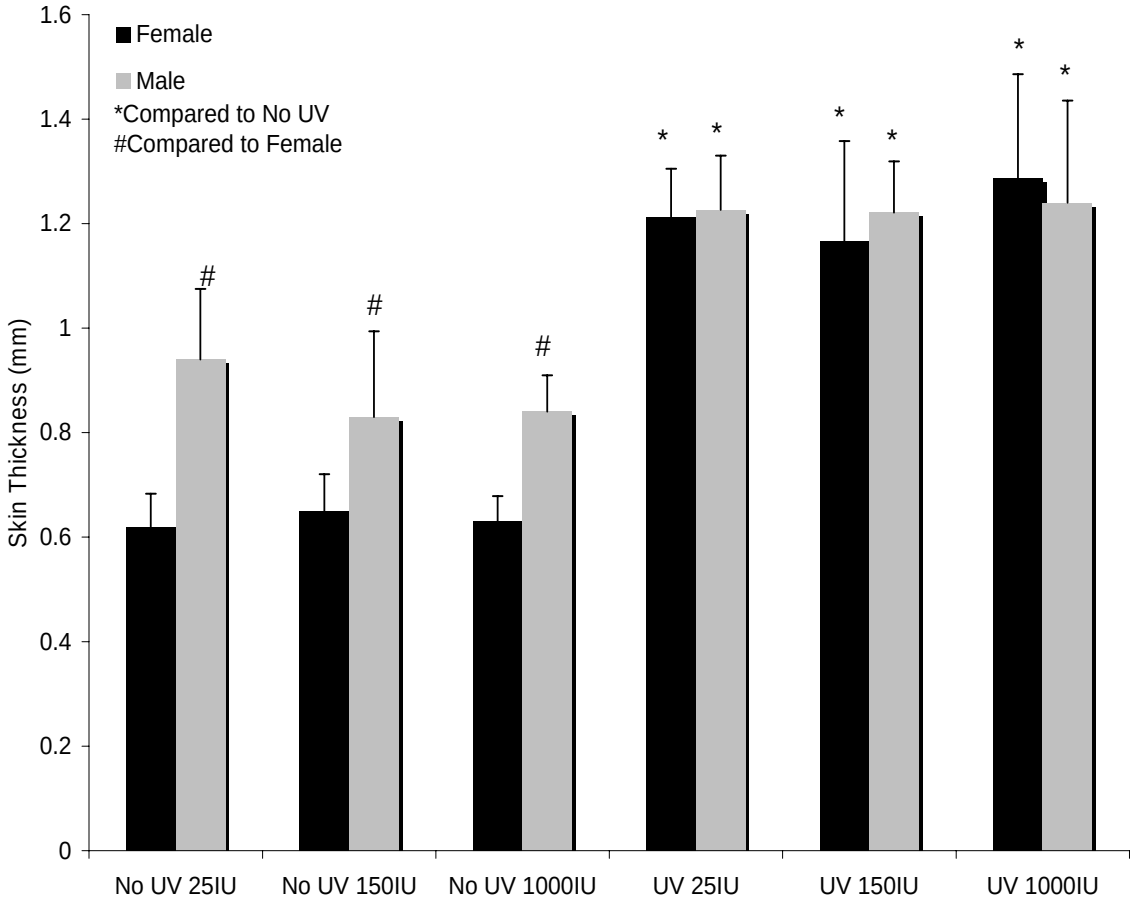

Figure 3. Dietary vitamin D supplementation effects on acute UVB induced edema. Male & female mice being fed 1 of 3 vitamin D containing diets (n=15 per diet) were exposed to 2240J/m^2 of UVB, sacrificed at 48h following exposure, and skin fold thickness was measured.

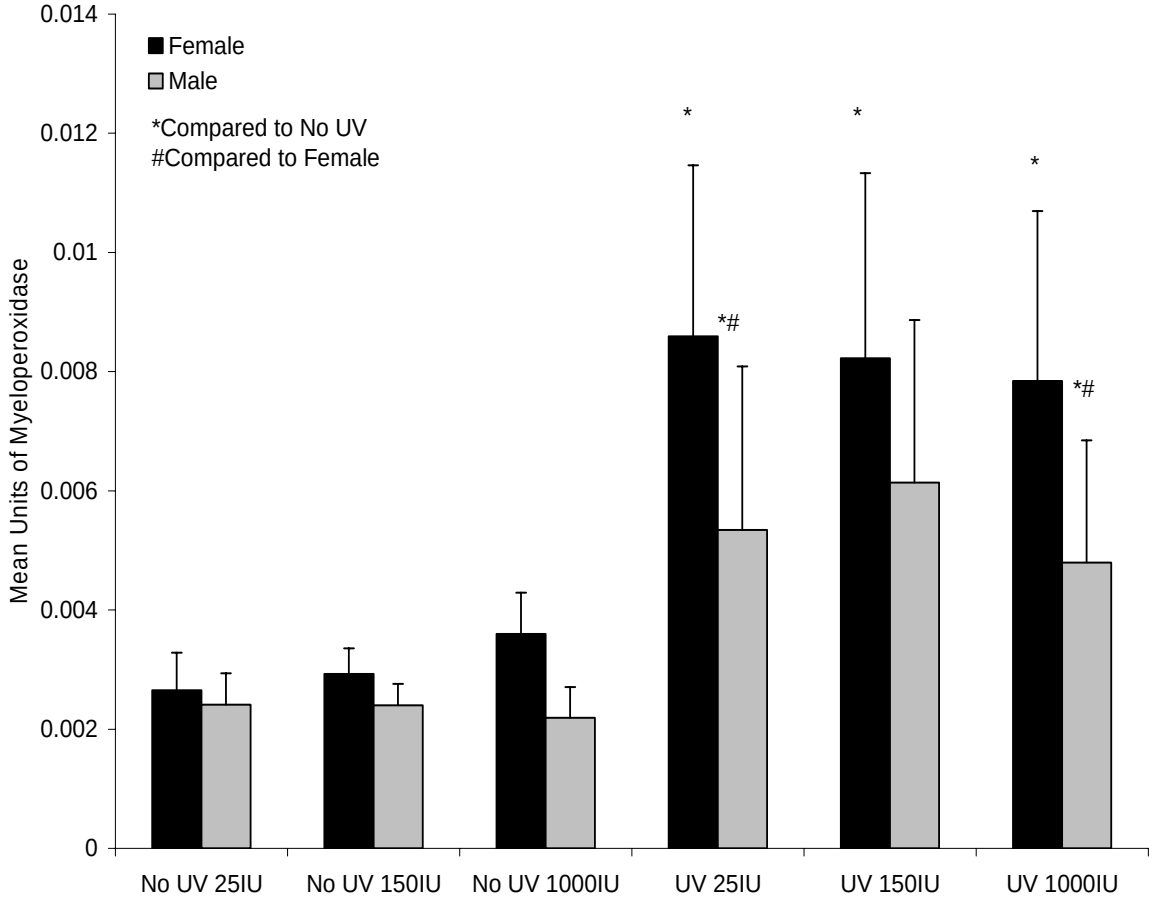

Figure 4. Dietary vitamin D supplementation effects on UVB induced myeloperoxidase activity. Myeloperoxidase activity was used to estimate the extent of inflammation in the skin of both male & female mice following 4 weeks of vitamin D supplementation and acute UVB exposure.

Effects of vitamin D on epidermal and dermal tissue morphology

Histochemical analysis was performed to analyze the effects of ultraviolet radiation and dietary vitamin D supplementation on epidermal and dermal tissue morphology (Fig. 5A-F). Skin samples from each of the 150 acute study mice were formalin fixed, paraffin embedded, cut into 5 μm thick sections and stained according to the Masson's Trichrome procedure (Fig. 5A-F). Next, epidermal and dermal thickness (in mm) was measured using ImageJ computer software. Following exposure to UVB radiation, epidermal thickness significantly increased in all mice (Fig. 5E). Vitamin D did not affect epidermal thickness. As for dermal thickness, in all cases male mice exhibited a thicker dermal layer than female mice. However, neither UVB exposure nor dietary vitamin D altered dermal thickness (Fig 5F). Figures 5A-D are representative photomicrographs of female unirradiated control mice (Fig. 5A), female irradiated mice (Fig. 5B), male unirradiated control mice (Fig. 5C), and male irradiated mice (Fig. 5D). Note that in the Masson's Trichrome stain photomicrographs (Fig. 5A-D) the upper red layer is the epidermal layer of tissue, the blue layer beneath the epidermis is the dermis, the colorless layer containing intermittent red structures is the fatty-tissue layer, and the bottom red striated layer is the muscular portion of the skin.

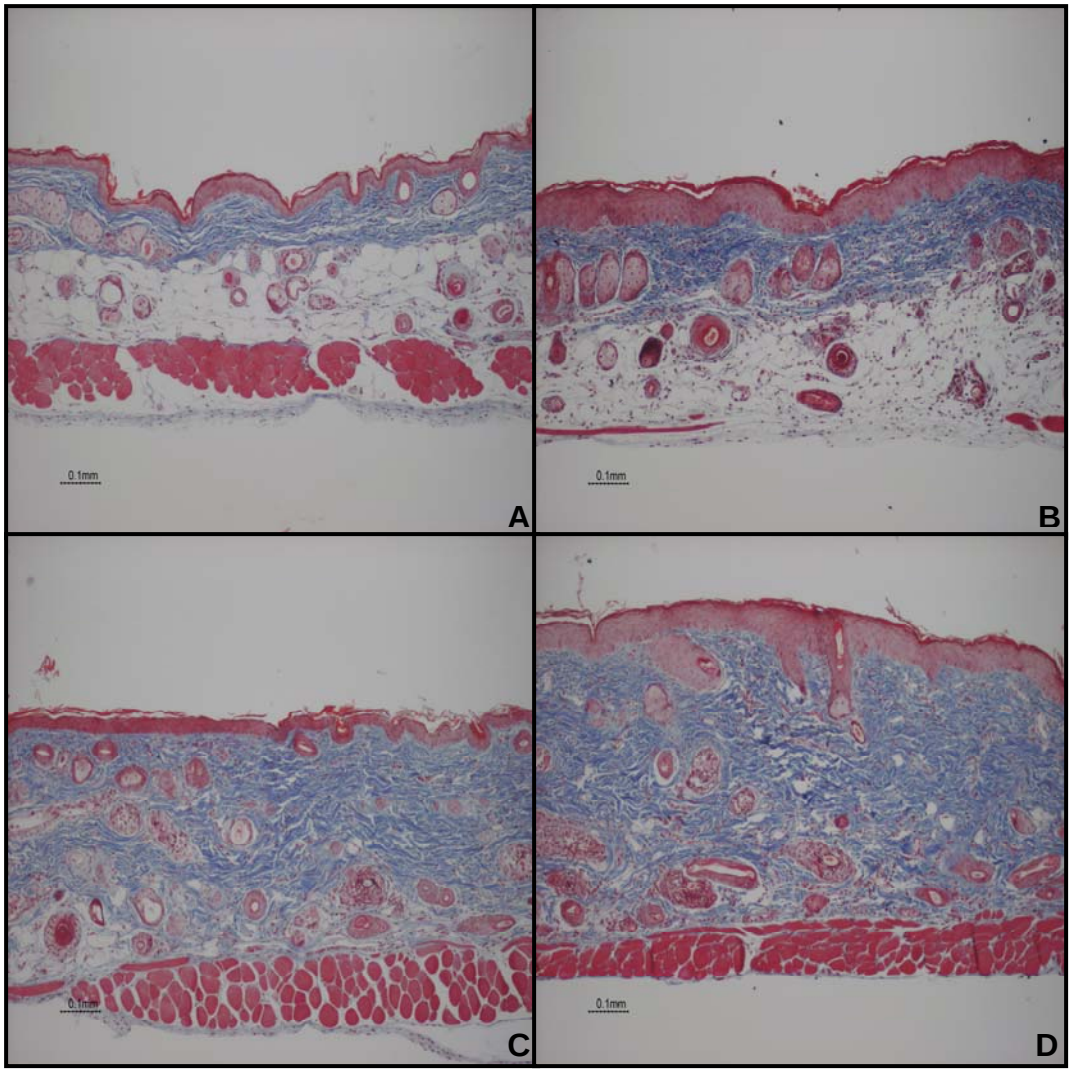

Figure 5A-D. Representative histological sections of Masson's Trichrome stained tissue in the female no UV group (A), female UV group (B), male no UV group (C), and male UV group (D). The upper red layer of skin is the epidermis, and located directly beneath the epidermis is the blue dermis. The dermis is separated from the lower red muscular layer by a fatty tissue layer which is not colored by the Masson's Trichrome dye. Photographs were taken at a magnification of 10x.

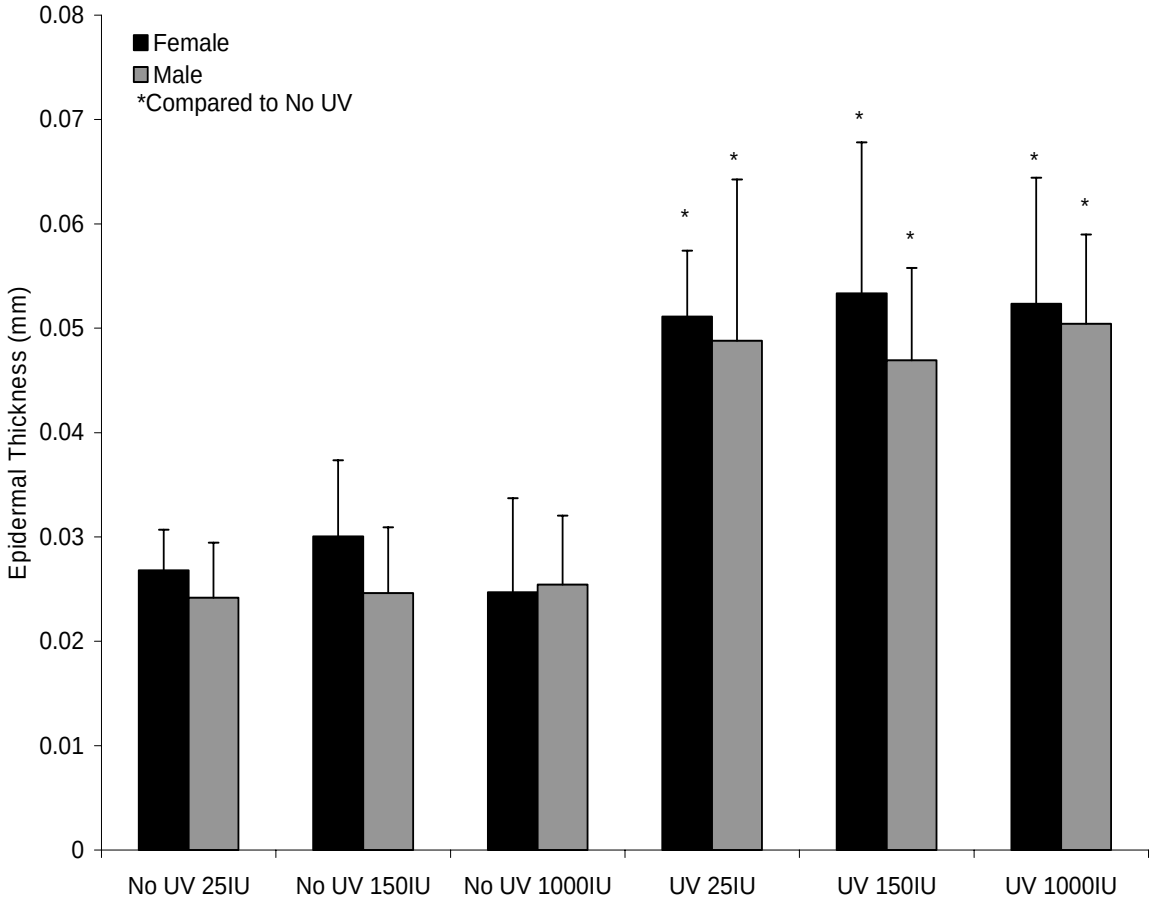

Figure 5E. Dietary vitamin D supplementation effects on epidermal tissue morphology. Skin samples from each of the 150 acute study mice were formalin fixed, paraffin embedded, cut into 5 µm sections and stained using the Masson's Trichrome procedure. Epidermal thickness was subsequently measured (in mm) using ImageJ software.

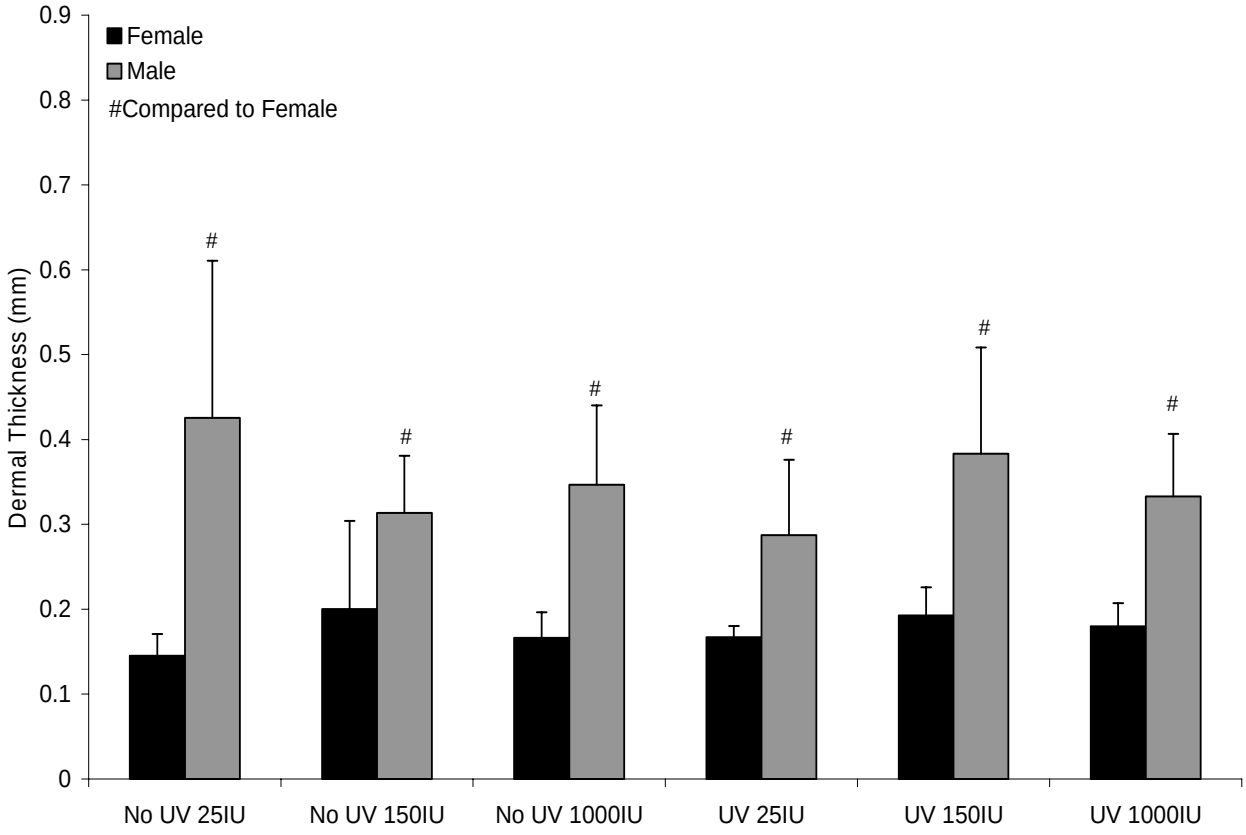

Figure 5F. Dietary vitamin D supplementation effects on dermal tissue morphology. Skin samples from each of the 150 acute study mice were formalin fixed, paraffin embedded, cut into 5 µm sections and stained using the Masson's Trichrome procedure. Dermal thickness was subsequently measured (in mm) using ImageJ software.

Effects of vitamin D on acute UVB induced direct DNA damage

To examine UVB-mediated direct DNA damage, immunohistochemical analysis to detect the presence of cyclobutane pyrimidine dimers was performed on skin samples from each of the 150 acute study mice (Fig. 6A-C). All nuclei, including positively stained nuclei, were counted, and the percentage of positive nuclei per total nuclei was calculated to indicate the amount of direct DNA damage present in the tissue samples. Figures 6A-B are representative photomicrographs of unirradiated control mice (Fig. 6A) and irradiated mice (Fig. 6B). In figure 6C, a trend can be observed in both male and female mice in the UV 150 IU/g and UV 1000 IU/g vitamin D containing diets. These groups exhibited decreased DNA damage in comparison to male and female mice in the UV 25 IU/g vitamin D diet group.

Effects of vitamin D on acute UVB p53

In the analysis of UVB induced global DNA damage, immunohistochemical analysis to detect the p53 tumor suppressor protein was performed on skin samples from each of the 150 acute study mice (Fig. 7A-C). All nuclei, including positively stained nuclei, were counted, and the percentage of positive nuclei per total nuclei was calculated to indicate the amount of global DNA damage present in the tissue samples. Figures 7A-B are representative photomicrographs of unirradiated control mice (Fig. 7A) and irradiated mice (Fig. 7B). Figure 7C reveals a potential vitamin D dose-dependent increase in tumor suppressor p53 to combat UVB induced global DNA damage in female mice, while male mice appear to be unaffected by varying vitamin D dosages in their diets.

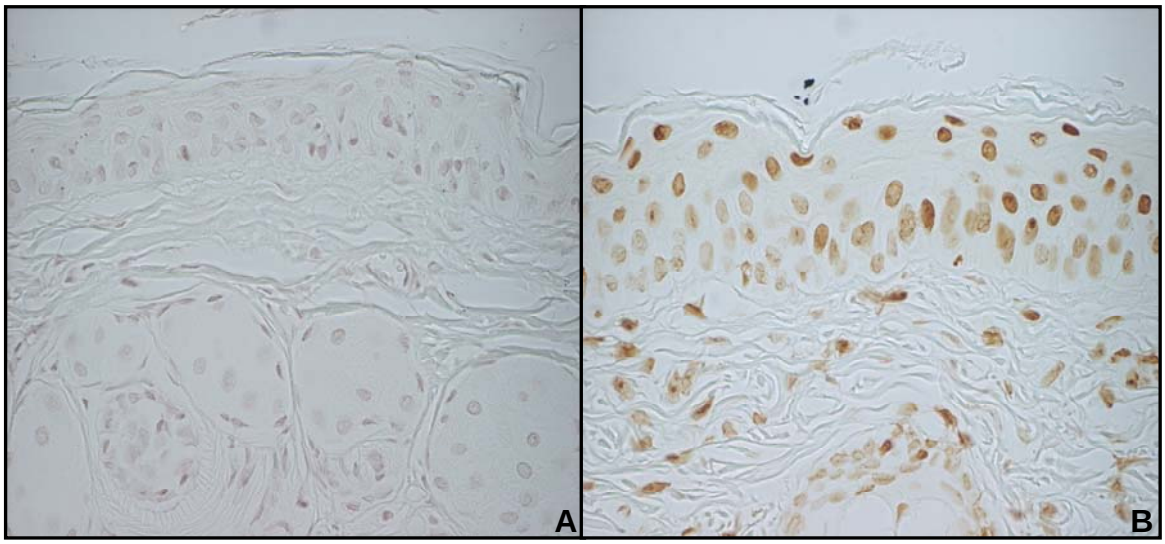

Figure 6A-B. Representative Immunohistologically stained sections of CPD stained tissue in the no UV group (A) and UV group (B). Cyclobutane pyrimidine dimer positive nuclei are stained brown. Photographs were taken at a magnification of 60x.

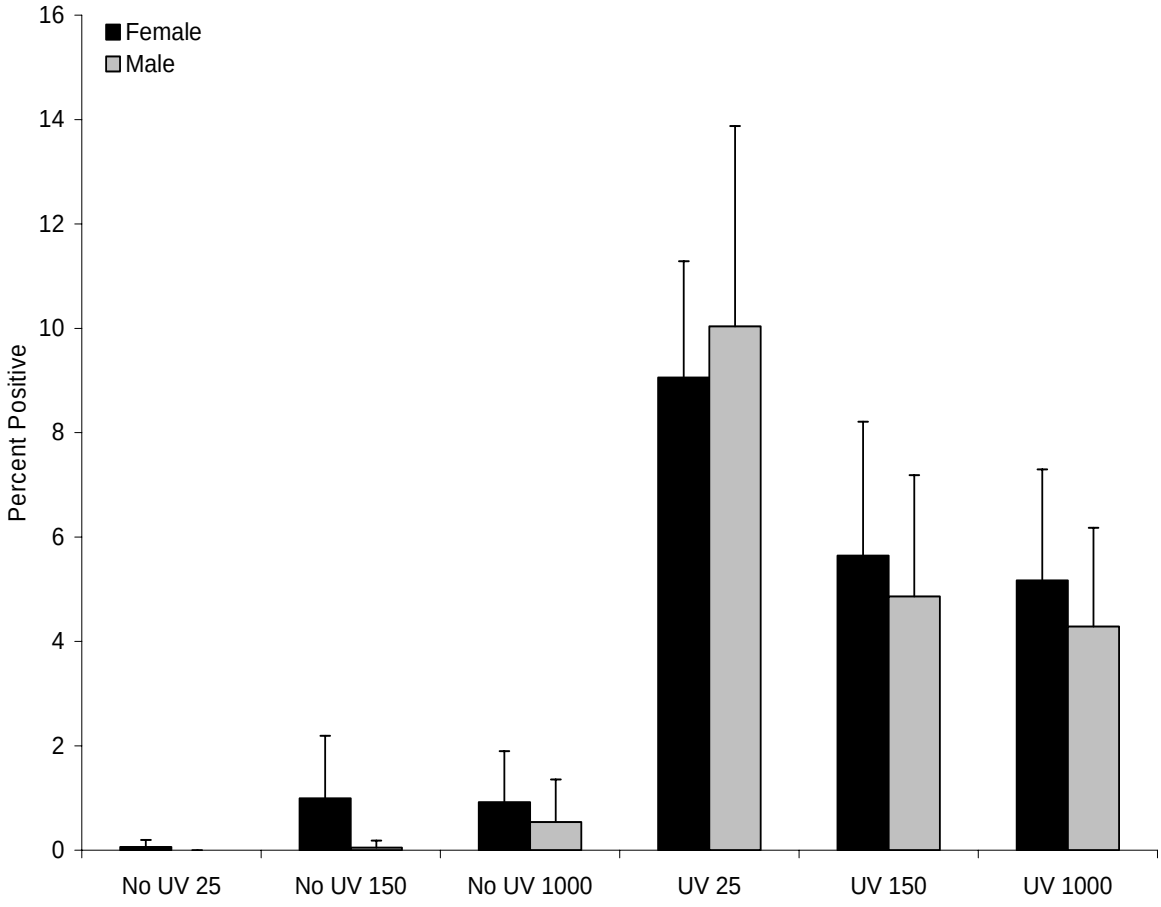

Figure 6C. Dietary Vitamin D Supplementation effects on UVB induced direct DNA damage. Immunohistochemical analysis was performed using skin samples from each of the 150 acute study mice. Measurement of the incidence of epidermal cyclobutane pyrimidine dimers allowed for quantification of direct DNA damage.

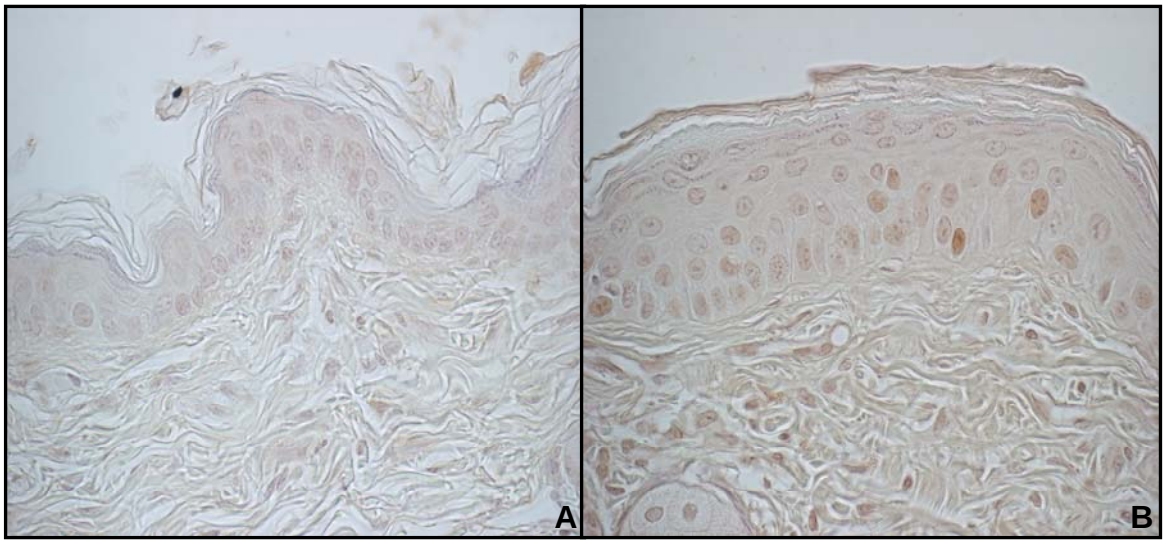

Figure 7A-B. Representative Immunohistologically stained sections of p53 stained tissue in no UV group (A) and UV group (B). P53 positive nuclei are stained brown. Photographs were taken at a magnification of 60x.

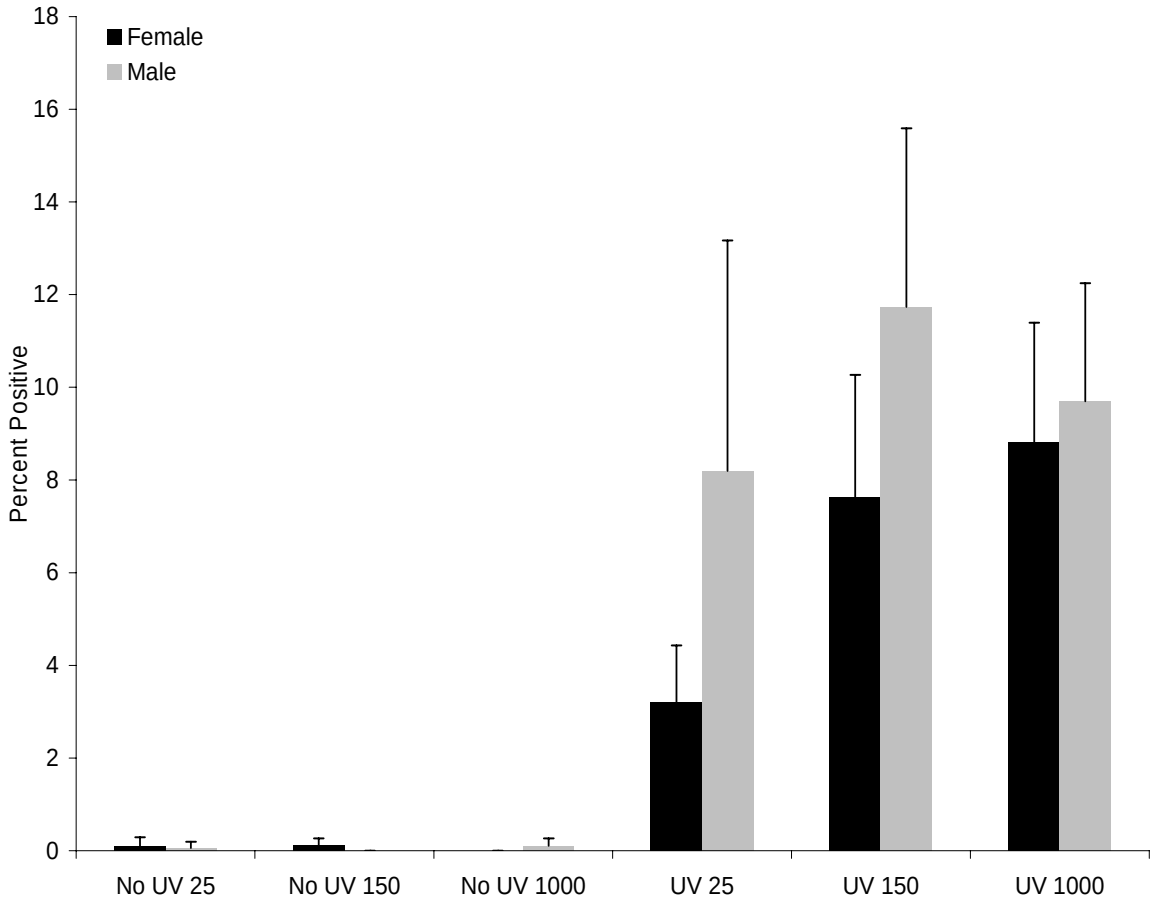

Figure 7C. Dietary Vitamin D Supplementation effects on UVB induced global DNA damage. Immunohistochemical analysis was performed using skin samples from each of the 150 acute study mice. Measurement of the presence of epidermal p53 allowed for quantification of global DNA damage.

Effects of vitamin D on chronic UVB induced tumorigenesis

In the chronic study, male and female mice were maintained on one of three vitamin D containing diets and were exposed to 2240 J/m^2 UVB 3 times weekly for 25 weeks. The first skin tumors were observed at six weeks following the initial UVB treatment, and tumor size (length x width) was measured weekly with digital calipers throughout the duration of the study. Average area per tumor (in mm^2) for each vitamin D diet group was calculated. Though no significant trend could be seen amongst female mice, vitamin D may have dose dependently increased the average area per tumor in male mice (Fig. 8). Similarly, upon examination of average tumor burden per mouse (in mm^2) in each vitamin D diet group, data revealed that female mice were not significantly affected by the concentration of vitamin D in their diets. Male mice, however, exhibited a potential vitamin D dose-dependent increase in average tumor burden (Fig. 9).

Effects of vitamin D on chronic UVB induced tumor grade

At the cessation of the chronic study, representative small, medium, and large tumors were obtained from each of the UVB irradiated mice, and board certified veterinary pathologist Dr. Donna Kusewitt determined the pre-cancerous or cancerous status of each of the selected tumors (Fig. 10). Though females do not appear to be affected by vitamin D levels in their diets, male mice exhibit a slight vitamin D dose-dependent increase in cancerous tumor numbers.

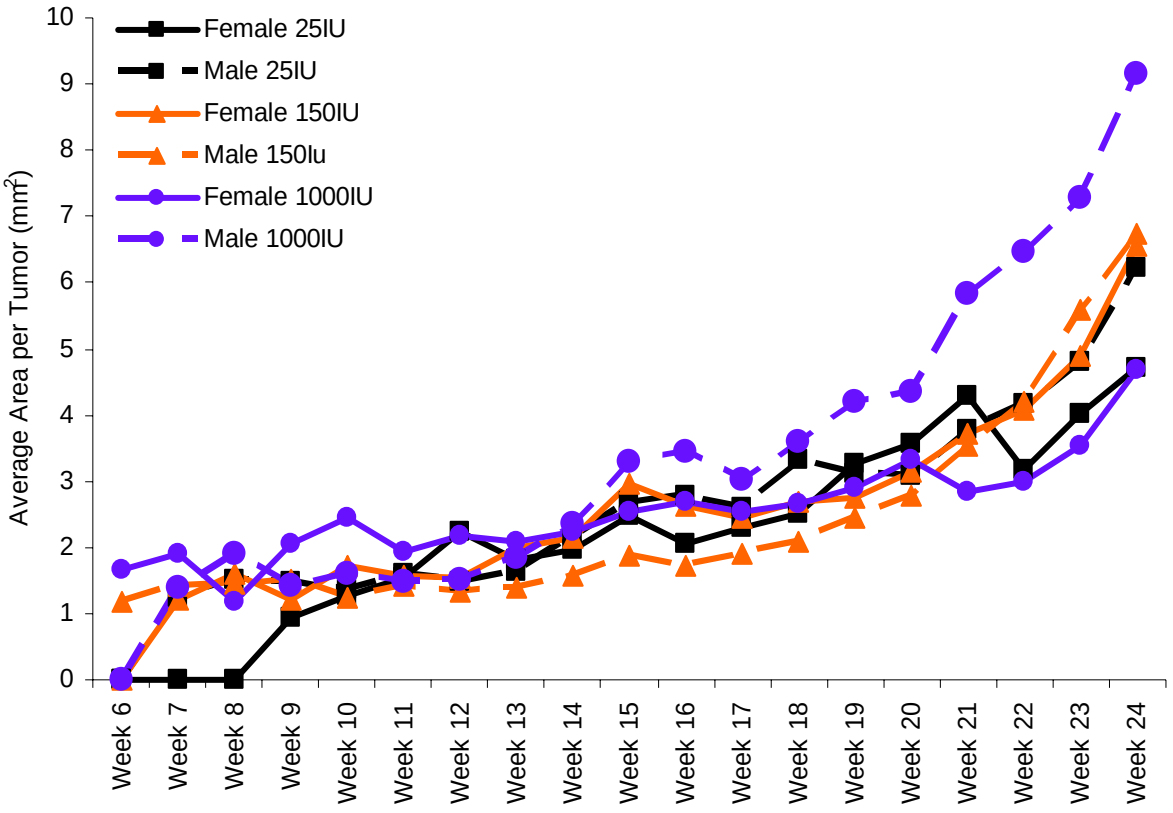

Figure 8. Dietary Vitamin D Supplementation effects on chronic UVB induced papilloma size. Male & female mice being fed 1 of 3 vitamin D containing diets (n=15 per diet) were exposed to 2240J/m2 of UVB 3X weekly for 25 weeks. Beginning during week 6 and throughout the duration of the study, tumor size was measured using digital calipers.

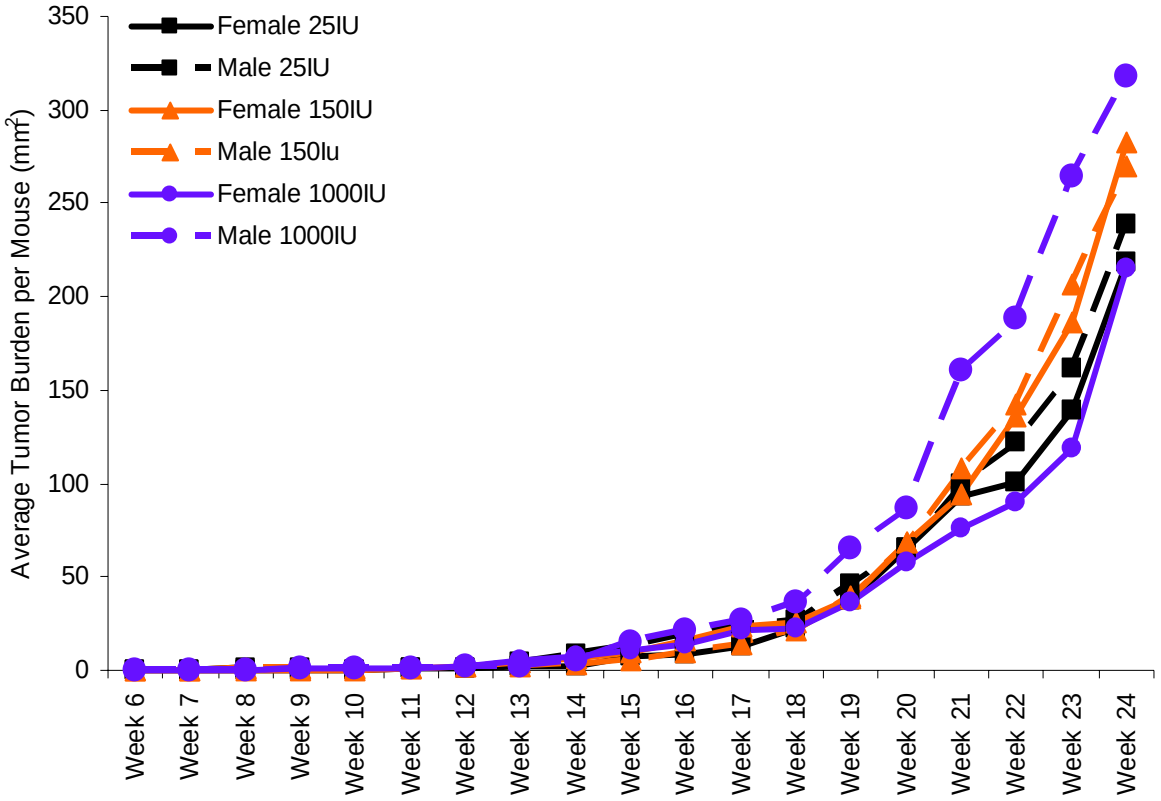

Figure 9. Dietary Vitamin D Supplementation effects on chronic UVB induced tumor burden. In the chronic study, average tumor burden per mouse was calculated for UVB irradiated mice which were fed one of 3 vitamin D containing diets.

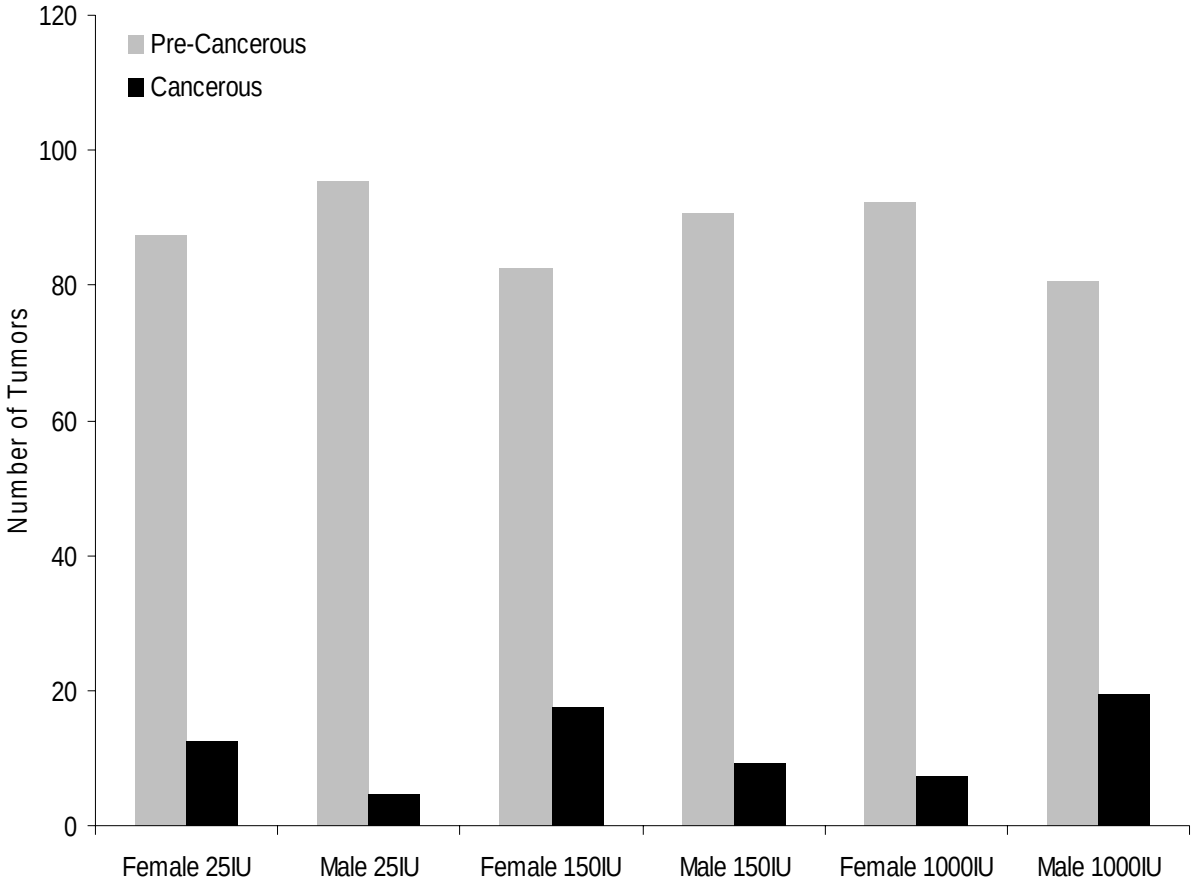

Figure 10. Dietary Vitamin D Supplementation effects on chronic UVB induced tumor grade. At the cessation of the chronic study, representative small, medium, and large tumors were obtained from each of the UVB irradiated mice. Tumor grade (pre-cancerous or cancerous) was determined by a board certified veterinary pathologist.

Discussion

The current acute and chronic studies have utilized a common non-melanoma skin cancer model, the Skh-1 hairless mouse, to analyze the effects of oral supplementation with vitamin D on UVB induced skin inflammation and carcinogenesis. Our laboratory has previously shown a clear link between UVB mediated cutaneous inflammation and skin tumor development. Specifically, we have found that if the inflammatory response is reduced through treatment with an anti-inflammatory drug, not only will acute UVB induced inflammation decrease, but skin tumor development will also be inhibited [19]. Accordingly, this vitamin D study was comprised of two components: an acute study to examine the UVB-mediated inflammatory response with varying vitamin D levels in the diet and a chronic study to analyze the effects of UVB mediated tumor development with varying vitamin D levels in the diet.

In the acute study, vitamin D did not appear to alter the inflammatory response. However, in measurements of edema formation and myeloperoxidase activity detection, as well as in histochemical analysis, exposure to UVB radiation resulted in increased inflammatory parameters. This effect was expected; as various studies have shown that exposure to UVB radiation elicits an immune response in the form of inflammation [20]. Additionally, the effects of acute UVB induced inflammation were examined through immunohistochemical analysis of direct DNA damage and global DNA damage. In immunohistochemical detection of cyclobutane pyrimidine dimers to quantify direct DNA damage, both male and female mice being fed the 150 IU/g and 1000 IU/g vitamin D diets exhibited a potential dose-dependent decrease in the observed amount of direct DNA damage. This is in accordance with prior in vitro studies which have revealed that the occurrence of

cyclobutane pyrimidine dimers decreases in irradiated skin cells treated with 1,25 dihydroxy vitamin D_3 in medium versus the vehicle [21]. These results imply that orally delivered vitamin D may demonstrate a sunscreen effect.

In vitro studies have shown that as direct DNA damage decreases, the levels of p53 in nuclei tend to be greater and a decrease in nitric oxide products also occurs [21]. Our study found through immunohistochemical analysis of the p53 tumor suppressor gene, a slight dose-dependent increase in p53 in female mice, indicating a greater concentration of anti-carcinogenic protein present within the cells. In addition to direct DNA damage, UV exposure also can induce indirect DNA damage via the production of reactive oxygen species. Therefore to further examine the effects of vitamin D on DNA damage, in future studies, immunohistochemical analysis to determine the levels of the oxidative adduct 8-oxo-deoxyguanosine and reveal levels of oxidative DNA damage must be performed. Additionally, future studies may benefit from measuring both systemic and cutaneous levels of vitamin D in order to discover the impact of the dietarily administered vitamin D on overall vitamin D levels in the mice. This will aid in determining the quantity of vitamin D in the diet necessary to obtain the most beneficial protection against the potential carcinogenic effects of ultraviolet light exposure.

In contrast to the results observed pertaining to DNA damage in the acute study, the chronic study revealed that increasing vitamin D levels via oral supplementation may negatively affect tumor development in male mice. No previous studies have analyzed the effects of dietary administration of vitamin D on chronic UVB induced skin carcinogenesis; however, it has been demonstrated that topical vitamin D application may result in a decrease in chemically induced skin cancer formation [22]. Conversely, through analysis of tumor size

measurements obtained over a 19 week period, it appeared as though average tumor size and tumor burden per mouse increased in male mice as the dosage of vitamin D in the diet became greater. This preliminary data suggests that vitamin D may act as a carcinogen in regards to skin tumorigenesis. To obtain a full picture of the role of vitamin D in carcinogenesis, further studies must be done to delve into the potential differential effects of vitamin D on tumor formation and progression in the genders.

By conducting both an acute and chronic study, we were able to closely look at the UVB mediated inflammatory response as well as the process of chronic UVB mediated tumorigenesis and the role of orally administered vitamin D in non-melanoma skin cancer. These studies consisted of a sub-physiologic 25IU/g vitamin D diet, a physiologic 150 IU/g vitamin D diet, and a supra-physiologic 1000 IU/g vitamin D diet as determined by the Research Diet company. Mice were fed one of the three vitamin D containing diets; however, no mice in the study were totally deprived of vitamin D or given a typical chow diet. These comparisons will be carried out in the future. Also, our understanding of the role of vitamin D in cancer will be greatly enhanced once researchers are able to reveal the mechanism by which vitamin D acts on cells in the body and reduces/enhances carcinogenesis and develop a better way to measure both circulating and tissue specific levels of active vitamin D. With the data gleaned in this study, coupled with further developments concerning vitamin D, we hope to discover the true action of this essential nutrient and potentially utilize its function in the protection against the most common cancer in the world, non-melanoma skin cancer.

Literature Cited

1. American Cancer Society: http://www.cancer.org, (2006).
2. National Cancer Institute: http://www.cancer.gov, (2008).
3. J. G. Chen, et al., Cost of nonmelanoma skin cancer treatment in the United States, *Dermatol. Surg.*, December 2001, 27(12), pp. 1035—1038.
4. P. Boukamp, Non-melanoma skin cancer: what drives tumor development and progression?, *Carcinogenesis*, October 2005, 26(10), pp. 1657—1667.
5. T. M. Oberyszyn, Non-melanoma skin cancer: Importance of gender immunosuppressive status and vitamin D, *Cancer Letters*, March 2008, 261(2), pp. 127—136.
6. F. R. de Gruijl, Photocarcinogenesis: UVA vs. UVB, *Methods Enzymol*., 2000, 319, pp. 359—366.
7. P. de Haes, et al., 1,25-Dihydroxyvitamin D_3 and analogues protect primary human keratinocytes against UVB-induced DNA damage, *J. Photochem. Photobiol. B.*, February 2005, 78(2), pp. 141—148.
8. B. A. Gilchrest, Sun exposure and vitamin D sufficiency, *Am. J. Clin. Nutr.*, August 2008, 88(2), pp. 570S—577S.
9. F. J. Duncan, et al., Topical treatment with black raspberry extract reduces cutaneous UVB-induced carcinogenesis and inflammation, *Cancer Prev. Res.,* July 2009, 2(7), pp. 665—672.
10. D. E. Brash, et al., Sunlight and sunburn in human skin cancer: p53, apoptosis, and tumor promotion, *J Investig Dermatol Symp Proc.*, April 1996, 1(2), pp. 136—142.
11. J. Martin, et al., Macrophage migration inhibitory factor (MIF) plays a critical role in pathogenesis of ultraviolet-B (UVB) -induced nonmelanoma skin cancer (NMSC), *FASEB J.*, March 2009, 23(3), pp. 720—730.
12. J. Reichrath, The challenge resulting from positive and negative effects of sunlight: how much solar UV exposure is appropriate to balance between risks of vitamin D deficiency and skin cancer?, *Prog. Biophys. Mol. Biol*., September 2006, 92(1), pp. 9—16.
13. C. F. Casey, et al., Vitamin D Supplementation in Infants, Children, and Adolescents, *Am. Fam. Physician,* March 2010, 81(6), pp. 745—748.
14. M. F. Holick, Sunlight, UV-radiation, vitamin D and skin cancer: how much sunlight do we need?, *Adv. Exp. Med. Biol*. 2008, 624, pp. 1—15.
15. J. Reichrath, Vitamin d and the skin: an ancient friend, revisited, *Exp. Dermatol*., July 2007, 16(7), pp. 618—625.
16. M. M. Ali, V. Vaidya, Vitamin D and cancer, *J. Cancer Res. Ther*., October-December 2007, 3(4), pp. 225—230.
17. J. Moan, A.C. Porojnicu, A. Dahlback, R.B. Setlow, Addressing the health benefits and risks, involving vitamin D or skin cancer, of increased sun exposure, *Proc. Natl. Acad. Sci. USA*, 15 January 2008, 105(2), pp. 668—673.
18. S. Adams, T.L. Clemens, J.A. Parrish and M.F. Holick, Vitamin-D synthesis and metabolism after ultraviolet irradiation of normal and vitamin-D-deficient subjects, *N. Engl. J. Med*., 1982, 306, pp. 722—725.
19. T. A. Wilgus, Inhibition of cutaneous ultraviolet light B-mediated inflammation and tumor formation with topical celecoxib treatment, *Mol. Carcinog.*, October 2003, 38(2), pp. 49—58.

20. J. M. Thomas-Ahner, Gender differences in UVB-Induced Skin Carcinogenesis, Inflammation, and DNA Damage, *Cancer Res.*,1 April 2007, 67(7), pp. 3468—3474.
21. R. Gupta, Photoprotection by 1,25 Dihydroxyvitamin D_3 Is Associated with an Increase in p53 and a Decrease in Nitric Oxide Products, *J. Invest. Dermatol.,* 2007, 127, pp. 707—175.
22. A. W. Wood, 1 alpha, 25-Dihydroxyvitamin D_3 inhibits phorbol ester-dependent chemical carcinogenesis in mouse skin, *Biochem. Biophys. Res. Commun.,* 31 October 1983, 116(2), pp. 605-611.